The Bacterial Basis of Biofouling: a Case Study

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For billions of years bacteria have profusely colonized all parts of the oceans and formed biofilms on the benthos. Thus, from their onset, evolving marine animals adapted to the microbial world throughout their life cycles. One of the major adaptations is the use of bacterial products as signals for recruitment by larvae of many species in the seven invertebrate phyla that make up most of the biofouling community. We describe here investigations on the recruitment biology of one such species, the circum-tropical serpulid polychaete Hydroides elegans. Insights gained from in-depth studies on adults and larvae of H. elegans include: apparently constant transport of these biofouling worms on the hulls of ships maintains a globally panmictic population; larvae complete metamorphosis with little or no de novo gene transcription or translation; larvae of this tube-worm settle selectively in response to specific biofilm-dwelling bacterial species; and biofilms provide not only a cue for settlement sites, but also increase the adhesion strength of the settling worms’ tubes on a substratum. Although biofilm-bacterial species are critical to larval-settlement induction, not all biofilm elements are inductive. Because studies on chemoreception systems in the larvae failed to find evidence for the presence of common chemoreceptors, we focused on the bacterial cues themselves. To do this, we studied a widely distributed and strongly inductive biofilm bacterium, Pseudoalteromonas luteoviolacea. Using molecular manipulations of its genome, we learned that it produces complex clusters of bacteriocins, multi-protein structures evolutionarily derived from phage-tail elements, which induce metamorphosis of H. elegans. But large questions remain: how do these complex structures bring about induction? Do larvae of other invertebrate species that settle in response to P. luteoviolacea also metamorphose in response to its bacteriocins? Do all inductive bacterial species produce bacteriocins? As a whole, the described studies on the recruitment of Hydroides elegans demonstrate the importance of in-depth investigations of model species for understanding the problem of biofouling as well as the ubiquity of essential animal-bacterial interactions in the sea.

Keywords: larval settlement, biofilms, Hydroides elegans, metamorphosis, biofouling

Introduction

Bacteria have proliferated in all of the world’s seas and oceans for most of Earth’s history, i.e., for about 3.5 billion years (Noffke et al., 2013). For roughly 1.75 billion years, bacteria had the seas to themselves, evolving, diversifying intensively and filling every possible marine environment (Knoll, 2004). Not only were bacteria abundant, but they surely were the food of the first eukaryotes as they evolved during the last 1.75 billion years. Evidence is now strong that the evolution of animals began only about 700 – 800 million years ago (Knoll, 2004), and there can be little doubt that the first benthic marine animals must have interacted intensively with the ubiquitous bacteria in the seas. How and when complex life histories involving biphasic life cycles and pelagic larvae evolved aren’t precisely known, but when they did evolve, larvae could not have approached any surface in the sea without encountering a dense bacterial film with which they had to interact in order to settle and metamorphose. It appears highly likely that these early larvae utilized chemical/biological cues from biofilm bacteria as signals for the suitability of particular habitats, a practice still present among larvae of most extant marine invertebrate species today (Hadfield, 2011).

Dependence of contact with marine biofilms, generally, or specific biofilm-bacterial species has been noted for members of every major phylum of marine invertebrate animals, including the seven phyla that characterize nearly all marine biofouling communities (Table 1) (Hadfield and Paul, 2001). Bacteria-associated attachment of algal spores has also been recorded (Joint et al., 2007). Our understanding of larval-bacterial interactions has increased many fold over the last 25 years, especially through in-depth investigation of specific “model species,” reared in the laboratory, for which developmental, sensory and ecological information has been gained. While these studies include many species of sponges (Porifera), hydrozoans and corals (Cnidaria), polychaetes (Annelida), bryozoans (Ectoprocta), mussels and oysters...
(Mollusca), barnacles (Arthropoda) and tunicates (Urochordata) (Table 1), a few species stand out. The barnacle Amphibalanus (Balanus) amphitrite and the bryozoan Bugulaneritina have provided numerous insights into the understanding of larval responses to bacterial films, especially, for the barnacle, with regard to sensory perception of settlement cues (Gohad et al., 2012, Maruzzo et al., 2012, Maruzzo et al., 2011). In this paper, we focus on the tropical biofouling serpulid tubeworm Hydroides elegans. Since we began studying the recruitment processes of H. elegans in 1990, the species has been mentioned in more than 1,500 scientific papers and is a major topic in at least 84, as indicated by inclusion of the species name in paper titles. In this review, we focus principally on the contributions of our University of Hawaii laboratory on the development of H. elegans as a research model organism, while emphatically acknowledging that other laboratories have made important contributions to understanding of the biology of the species, especially that of Dr. Pei-Yuan Qian at Hong Kong University of Science and Technology, whom we introduced to H. elegans in the mid-1990s.

Choosing the species

In 1990, researchers in our laboratory initiated studies of the biofouling community in Pearl Harbor, Hawaii, a port that has served as a major United States naval base for more than 125 years. Similar to other such bays around the world, Pearl Harbor had long accumulated a large element of the global, warm-water biofouling community, carried to it on the hulls of ships, barges and structures like floating dry-docks (Carlton and Eldredge, 2009). We learned early on that one of the major problems experienced by the U.S. Navy in Pearl Harbor was the accumulation of calcareous tubes of the polychaete worm Hydroides elegans Haswell (1883) on the hulls of ships and other structures (Figure 1). Because we quickly found the species on test panels submerged in the harbor, we immediately focused some of our efforts on understanding the recruitment biology of H. elegans. It turned out to be an exceptionally facile species for studies of its life history in the laboratory. Our first attempts to observe larval development and settlement of H. elegans were successful, enabling us to begin many years of experimentation that have led to a current understanding of the recruitment processes of H. elegans that is arguably among the most extensive for marine invertebrate species.

Initial observations of larval settlement of H. elegans led us to suspect that the larvae were responding to fouled surfaces; that is, surfaces that were obviously coated with a film of bacteria, diatoms and other microorganisms. These surfaces were quickly colonized by settling larvae of H. elegans while clean ones were not. Finding the species both easy to rear and induce to settle led to a series of controlled laboratory experiments from which we learned the critical life history characteristics for the worm that were essential to utilizing it extensively in the laboratory. H. elegans, as collected from the field, is dioecious and free spawning; i.e., sexes are separate, fertilization is external and the larva develop in the plankton. At least some individuals are protandric, maturing first as males and later transforming into females. The species develops rapidly into planktrotrophic-trochophore larva within a day that develop into metamorphically competent nectochaete larvae in 4 – 5 days at 25°C (Figure 2) (Hadfield et al., 1994, Carpizo-Ituarte and Hadfield, 1998). It is at this point that settlement / metamorphic induction is necessary. In a first set of experiments, we demonstrated that the percentage of a cohort of larvae of H. elegans that settled on a surface was strictly proportional to the time that the surface had been immersed in flowing seawater (Hadfield et al. 1994). Furthermore, when we counted the abundance of various types of microorganisms that were on the surfaces that had been immersed for different periods, the tightest correlation with larval settlement abundance was the density of short, rod-shaped bacteria. From this, we concluded that it was bacteria in this category to which the larvae responded by attaching and rapidly secreting a membranous primary tube within which they completed metamorphosis (Figure 3). Subsequent research confirmed this finding, adding that many different bacterial species isolated from inductive biofilms induced larval settlement of H. elegans (Unabia and Hadfield, 1999). Information on the handling of H. elegans as an experimental organism in the laboratory was summarized by Nedved and Hadfield (2009).

Larval responses in Hydroides elegans

While continuing to explore the variety of bacteria that induce settlement in the tubeworm, we began to extensively investigate the mechanisms by which larvae recognize a bacterial settlement cue and translate that cue into a morphogenetic process. Holm et al. (1998) investigated the nature of the cellular receptor that larvae might use to detect specific bacterial substances, expecting to find
Fig. 1 – Adults of the serpulid tubeworm Hydroides elegans. A. Several adult worms on a submerged surface. B. Accumulation of tubes of Hydroides elegans on a surface after 1-month submersion in Pearl Harbor, HI. Scale bars = 1 cm.

Fig. 2 - Larval development in Hydroides elegans at 25 °C. A. Lateral view of a trochophore larva (12 h post-fertilization). B. Dorsal view of a nectochaete larva (~120 h post-fertilization). Scale bars = 50 μm.
evidence that a G-protein-coupled receptor would be found, because this type of receptor has been implicated in most instances of chemosensory reception in taste and smell (Buck and Axel, 1991, Ngai et al., 1993). However, the results were negative in experiments applying pharmacological substances that should either stimulate G-protein coupled receptors, and therefore induce larval settlement in the absence of a bacterial film, or inhibit settlement in the presence of a biofilm. Holm et al. (loc. cit.) concluded that bacterial-signal reception and transduction in larvae of *H. elegans* must rely on a different receptor type, with many other known possibilities. Experiments were also performed to provide greater understanding of the way in which the bacterial cue stimulated metamorphosis. We had long known that 20 – 50 mM potassium chloride added to natural seawater would stimulate metamorphosis in other larval types (e.g., Yool et al., 1986), a finding that was confirmed in *H. elegans*. It was additionally found that exposing the larvae to a 3-hour pulse of 10 mM CsCl added to seawater was inductive (Carpizo-Ituarte and Hadfield, 1998). We concluded from this that the bacterial cue, in some way, activates the larval nervous system, which, in turn, stimulates the morphogenetic responses specific to different tissues.

The extent to which the initial settlement and processes of metamorphosis are dependent on *de novo* gene action or upon translation of preexisting messenger RNAs in larvae of *H. elegans* was investigated by rearing larvae in the presence of both transcriptional and translational inhibitors and observing whether or not larvae settled and completed metamorphosis (Carpizo-Ituarte and Hadfield, 2003). The surprising result with either set of inhibitors was that larvae settled normally, secreted primary tubes and advanced through metamorphosis to the stage when the buds of the branchialradioles typically begin to elongate, and then arrested. From this we concluded that little, if any, *de novo* genetic transcription or translation was necessary for settlement and the initiation of metamorphosis in *H. elegans*.

Based on experience in studies of a number of different marine larval types, including *H. elegans*, we developed theoretical analyses of larval development noting: (1) over the prior 20 – 30 years there had been increasing awareness that embryonic development for most larvae continues during the planktonic phase to the establishment of "competence," defined as the capacity to undergo metamorphic transformation while retaining the larval morphology and biology until a specific stimulus is encountered; and (2) a large body of evidence revealed that larvae do not so much
“choose” the sites where they settle, but rather are stimulated or triggered to settle by more-or-less specific stimuli from specific environments. In the latter case, the vocabulary became “settlement or metamorphic induction,” rather than larval choice (Hadfield, 1998, 2000). We further commented on the broad phylogenetic occurrence of the competent state in invertebrate larvae, the condition wherein most or all juvenile structures are already present in the larvae and that it had apparently evolved multiple times (Hadfield et al., 2001). The tubeworm *Hydroides elegans* exemplifies this well. By the time the larva of this species is metamorphically competent, it has three well developed, setae-bearing segments and a complete gut. The major stages of metamorphosis of *H. elegans* are: (1) attachment to a firm, typically biofilmed surface; (2) secreting a membranous primary tube from the larval epidermis; (3) shedding the ciliarytrochal bands that provide the mechanism for both larval swimming and filter feeding; and, (4) transforming the anterior, pre-trochal region of the body into the branchial crown of tentacles (Carpizo-Ituarte, 1998, 1998, Nedved and Hadfield, 2009).

Although the first papers from the our laboratory had established the necessity for only a marine biofilm to stimulate settlement of *H. elegans*, other laboratories reported on a role for gregarious factors in stimulating settlement of the species (Bryan et al., 1997). To more deeply investigate this question, we carried out laboratory tests wherein we exposed competent larvae to surfaces bearing either living *H. elegans* in their tubes, tubes of *H. elegans* from which the adults had been removed, or tube mimics consisting of 10-mm long pieces of non-toxic, polyethylene surgical tubing 1.09 mm in diameter (Intramedic #7405, Becton Dickinson & Co.), i.e., about the same diameter as the tubes of adult *H. elegans*, with or without a biofilm (Walters et al., 1997). We found that larval settlement patterns were essentially the same on all three test surfaces. In addition, Walters et al. (1997) suspended plates with similar distributions of worms, empty worm tubes or tube mimics in Pearl Harbor for six days and afterwards noted both the numbers and locations to which

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larvae had recruited to the plates. Again, the result was that larvae of *H. elegans* settled in very similar locations on all three, and more abundantly in the crevices formed between the tubes and the plate surfaces, most probably due to larval selection of biofilmed surfaces on the down-stream sides of the tubes in flowing water. That is, the experiments demonstrated no gregarious preference by the settling larvae; only the presence of a biofilm was of critical importance.

**Bacterial induction of settlement and metamorphosis in *Hydroides elegans***

Although the nature of larval cell-surface receptors for bacterial settlement cues continues to evade us, we have made significant progress in analyzing the bacterial cues themselves. A first step was isolation of bacterial strains from marine biofilms that were strongly inductive for metamorphosis of larvae of *H. elegans* (Unabia and Hadfield, 1999). We found that a wide variety of bacteria from many bacterial groups had that capacity. Next, a small set of strongly inductive and non-inductive bacteria was selected for in-depth characterization, first by obtaining their identities from sequences of the 16S ribosomal RNA gene, and then by examining their relative densities in mono-specific biofilms (Huang and Hadfield, 2003). Of four bacterial species analyzed in this study, two were found to be non-inductive and the two others inductive, one producing greater settlement and metamorphosis than the other. The inductive activity of the bacteria was not found to correlate with their taxonomic relationships. That is, one species of the genus *Cellulophaga*, *C. lytica*, induced settlement of larvae of *H. elegans* and another species in the same genus did not. The most inductive bacterial strain was identified as the species *Pseudoalteromonas luteoviolacea*. A bacterium species identified as *Flexibacter* sp. was non-inductive. Further research revealed that a congener of the most inductive species, *Pseudoalteromonas atlantica*, was non-inductive. Furthermore, Huang and Hadfield (2003) found that nearly equally inductive biofilms from *P. luteoviolacea* were less dense than biofilms made from only *C. lytica*; clearly, the quality of specific bacteria in a biofilm has greater importance in larval recruitment than simply the density of any given bacterial species.

Focusing on *P. luteoviolacea*, Huang et al. (2012) employed transposon mutagenesis to obtain two non-inductive strains of the bacterium; that is, biofilms made from either of two mutated bacterial strains failed to induce settlement of *H. elegans* under conditions identical with those in which the wild-type strain induced most larvae to settle and metamorphose. These two non-inductive strains were then subjected to intense sequence analysis using repeated PCR, often referred to as “genome walking,” to characterize the genes whose disruption had led to the loss of inductive capacity (Huang *et al.*, 2012). The two mutated genes were found to lie close together in a single large operon containing at least seven genes and separated by only a single open-reading frame. Subsequent sequencing of the entire genome of *P. luteoviolacea* allowed us to verify the gene sequences and their positions relative to one another. In-frame deletion mutants for the seven genes lying sequentially in the operon revealed that each of the first four was individually required for the inductive activity, and the last three were not. Further analyses indicated that differences in bacterial density or production of the extracellular polymeric substances (EPS) in biofilms from the mutant strains were not responsible for the lack of inductive capacity. Comparisons of the sequenced genes in the operon against known sequences in the NCBI protein database failed to provide any exact identities, although the first one has similarity to a hypothetical phage-tail protein and the second one has elements related to biofilm production in some bacteria. The third gene transcribes a protein that may function in cell adhesion. Most importantly, it was found that genes whose products are essential to the settlement-inducing capacity of *P. luteoviolacea* produce apparently structural proteins rather than elements that might simply be part of the mucus-rich EPS with which bacteria coat themselves in biofilms (Huang *et al.*, 2012).

The latest stage in our analysis of the inductive capacity of *P. luteoviolacea* included a more extended investigation of the genome of this bacterium, especially a search for additional elements of phage-tail systems identified in other bacteria and called bacteriocins (Shikuma *et al.*, 2014). Bacteriocins in several bacterial species are known to be employed to kill other, presumably competing, bacterial species (Michel-Briand and Baysse, 2002, Riley and Wertz, 2002). They are complex organelles composed of the products of multiple genes that employ the injection apparatus of bacteriophages to either perforate the cell membranes of target bacteria or to inject substances (toxins?) into other bacteria. Bacteriocins in a few species have been found to kill insects (e.g., Yang *et al.*, 2006). In *P. luteoviolacea*, we found additional genes for bacteriocin structures positioned upstream from the operon identified by
Huang et al. (2012). Again employing in-frame deletion mutation, we found three genes for phage-tail like structures to be essential for the bacterium to induce settlement of larvae of *Hydroides elegans* (Shikuma et al., 2014). A further analysis, employing the methods of electron cryotomography, revealed that single cells in a population of *P. luteoviolacea* synthesize great numbers of bacteriocins that are linked into highly organized arrays and released into the EPS by cell rupture. The bacteriocin clusters are apparently retained in the EPS of the biofilm and interact with larvae of *Hydroides elegans* to stimulate their settlement and metamorphosis (Shikuma et al., 2014).

While we now have a significantly greater knowledge of the characteristics of a bacterial species that is important in initiating biofouling by the tubeworm *Hydroides elegans*, we have as yet no understanding of how complex arrays of bacteriocins, derived from gene sets evolved to allow viruses to inject their DNA into living bacterial cells, cause larvae to settle. Do the bacteriocins inject a substance into the polychaete larvae that, in some manner, induces their settlement and metamorphosis? Or, do the bacteriocins simply interact with the membranes of sensitive cells on the larval surface, or perhaps cilia on such cells, puncturing them sufficiently to cause an ion flux that initiates an action potential that brings about metamorphosis? Data cited above on ionic induction of metamorphosis in larvae of *H. elegans* and many other species suggest this might be the case. Furthermore, we don’t yet know if other inductive bacteria also produce bacteriocin arrays that can induce settlement in *H. elegans* or if the larvae respond to different cues from different bacterial species. And, finally, we don’t know whether bacteriocin arrays in *P. luteoviolacea* are the element that brings about metamorphosis in coral larvae (Tran and Hadfield, 2011) and those of an echinoid in Australia (Huggett et al., 2006), both of which are known to settle in response to the same bacterium. The genus *Pseudoalteromonas* has been identified in many biofilms and implicated in the biology of other larval types (Hadfield, 2011, Tebben et al., 2011, Snee et al., 2014). Studies on the interactions of larvae from other phyla with bacteria, in general, and with species of *Pseudoalteromonas*, specifically, will be especially enlightening.

As noted above, *Hydroides elegans* has been recognized in biofouling communities in tropical and warm-temperate seas around the world. We will likely never know exactly where the species originated before its journeys across the seas on the hulls of ships began long ago. What we have been able to determine using genetic analyses is that *H. elegans* continues to be distributed circum-globally, surely on ship hulls, to maintain broadly interbreeding populations (Pettengill et al., 2003, Pettengill et al., 2007). Because it is so easily reared in the laboratory, Miles et al (2007) carried out extensive selection and inbreeding experiments for eleven generations to understand the capacity for natural selection to impact egg size. They found significant heritability for the trait, indicating “… substantial potential for egg size to respond to varying selective pressures…” with implications for variations in larval size and longevity.

The physical relationships between settling larvae of *H. elegans* and biofilms have been investigated in several ways. Zardus et al (2008) examined the surface-adhesion strength of primary and secondary tubes on clean and biofilmed surfaces. They did this by stimulating larvae to settle on natural biofilms accumulated on glass slides or on clean slides where they had been induced to settle by exposure to 10 mM cesium chloride in seawater. The slides bearing newly settled worms in primary tubes were then placed into a turbulent flow channel and exposed for four minutes to a wall-shear force of 50 pascals to measure their resistance to dislodgement, after which the slides were removed from the flow channel and remaining juveniles counted. The result was that significantly more juveniles remained on the biofilmed slides than on the clean ones. That is, the primary tubes of newly settled juveniles had stronger adhesion on biofilmed surfaces than on clean ones, which may shed some light on the reason for selective settlement on biofilms by larvae of *H. elegans* and other animals. Because larvae of some biofouling invertebrates have been reported to respond differentially to a range of surface-free energies, or wettabilities, we have examined the relationship between surface free energy, biofilm accumulation and settlement by *H. elegans* in the field and the laboratory (Huggett et al., 2009). The general result was that, after 10 days of submersion of a set of surfaces with a range of wettabilities, all the surfaces were equally, and highly acceptable for settlement by the larvae. That is, marine biofilm bacteria quickly form on surfaces with a broad range of free energies and thus provide an equal...
stimulus for larval settlement, a finding with significance for those who may place hope in developing foul-resistant coatings based on initial wettability characteristics.

Conclusions

*Hydroides elegans* has proven to be an important model system for the study of larval development and metamorphosis of marine invertebrate animals, comparable to a few other species such as the hydrozoan *Hydractinia echinata*, the bryozoan *Bugula neritina*, the oysters *Ostrea edulis* and *Amphibalanus* (Balanus) amphitrite urchin *Strongylocentrotus purpuratus*, the barnacle *Amphibalanus* (Balanus) amphitrite and the ascidian *Ciona intestinalis*. Importantly, *H. elegans* is a prominent member of the warm-water biofouling community around the world and thus knowledge of the species’ recruitment biology is illustrative for a large number of such species that recruit in response to bacterial species in the ubiquitous biofilms that coat all surfaces submerged in the sea. Finally, studies of the biology of *H. elegans* reveal it to be a particularly instructive example of the essential bacteria-animal interactions now recognized as critically important in the biology of all animals and universal in all of earth’s ecological settings (McFall-Ngai et al. 2013).

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